Appendix 16

(16)

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IMMUNOCHEMISTRY OF GROUPS A, B, AND C MENINGOCOCCAL POLYSACCHARIDE-TETANUS TOXOID CONJUGATES[1]

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The successful coupling of the meningococcal groups A, B, and C polysaccharides to tetanus toxoid to yield water soluble conjugates is described. Reactive aldehyde groups were strategically introduced into the terminal residues of the polysaccharides by the controlled periodate oxidation of the native groups B and C polysaccharides and of the group A polysaccharide previously modified by the reduction of its terminal reducing *N*-acetylmannosamine residue. This produced essentially monovalent polysaccharide molecules, which were subsequently covalently linked to tetanus toxoid by means of reductive amination. Although the groups A and C polysaccharides proved to be poor immunogens in rabbits and mice, their tetanus toxoid conjugates produced high levels of polysaccharide-specific antibodies in both animals. By contrast, even in the form of its tetanus toxoid conjugate, the group B polysaccharide failed to elicit homologous polysaccharide-specific antibodies in either animal; a major proportion of the antibodies actually produced had a specificity for the linkage area of the conjugate. This evidence is compatible with the hypothesis of the poor immunogenicity of the group B polysac-

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charide being structure related. Hyperimmunization of mice with the groups A and C polysaccharide-tetanus toxoid conjugates produced antisera with good bactericidal activity against their respective homologous organisms, and indicated the potential of these conjugates as potential human vaccines.

Despite the demonstrated success of the groups A and C capsular polysaccharides of *Neisseria meningitidis* in providing homologous serogroup immunity in humans (1), there still remain major problems associated with their use as human vaccines. First, the group B polysaccharide is only poorly immunogenic in man (2), and second, both the A and C polysaccharides are relatively poor immunogens in very young infants (3). This latter situation is highly undesirable because this section of the population experiences the highest incidence of these meningococcal infections (3). Therefore, in order to surmount these problems, it is necessary to enhance the immunogenicity of these polysaccharides by converting them to thymus dependant immunogens (4–8). One possible method of achieving these objectives is by the conjugation of these polysaccharides to a carrier protein. The feasibility of this approach has been well established in earlier work (4, 9, 10), although the coupling methods employed resulted in the formation of linkages having highly undesirable structural features for use in human vaccines.

More recently, the use of simple and efficient coupling techniques employing reagents that form linkages having more acceptable structural features have been reported (5–7, 11). Except for the EDC[3] method (12) employed by Svenson and Lindberg (11) on the large m.w. oligosaccharides obtained from Salmonella *O*-antigens, the 2 methods employed by Schneerson *et al.* (7) and Beuvery *et al.* (5, 6) to conjugate the respective larger molecular size *Haemophilus influenzae* type b and *N. meningitidis* group C polysaccharides to TT were based on the random activation of the functional groups of these polysaccharides. We have tried to extend the monofunctional group approach used in conjugating oligosaccharides (11, 13) to the larger molecular size meningococcal polysaccharides. This was achieved by the introduction of a terminally located free aldehyde group into the polysaccharide molecules, through which they could be specifically coupled to protein without activating the other functional groups on the polysaccharide. This procedure avoids cross-linking, minimizes the possibility of extraneous chemical modification of the polysaccharides, and results in better-defined immunogens.

MATERIALS AND METHODS

Materials. Crystalline BSA was obtained from Schwarz-Mann, Orangeburg, NY, and L(+) lysine monohydrochloride from Eastman Kodak Co. Rochester, NY. Neuraminidase (*Vibrio cholerae*) was obtained from Calbiochem-Behring Corp. La Jolla, CA, and tetanus toxoid (TT) was a gift from Dr. Marc Quevillon of the Institut Armand Frappier, Montreal, Québec, and was additionally purified using a Sephadex G-100 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS.[3] Only the fraction in the void volume of the column was used in the conjugation experiments. *N. meningitidis* strains 604A, 608B, and 2241C were obtained from the culture collection of the Laboratory Center for Disease Control, Ottawa, Ontario, and were grown in a chemically defined medium (14). The cetavlon-precipitated capsular polysaccharides were purified by extraction with hot buffered phenol and treatment with ribonuclease (15). The polysaccharides, modified polysaccharides, and oligosaccharides utilized in the conjugation experiments were of selected molecular sizes. This selection was achieved by the fractionation of the above preparations on the appropriate calibrated Sephadex G-100 or G-50 (for the group B polysaccharide) columns equilibrated with PBS. The selected fractions were then reapplied to the same respective columns. The column eluates were monitored using a Waters Associates differential refractometer (model R403). In each case the selected molecular size fractions constituted the major portion of the eluted polysaccharides, which in the case of the group A and B polysaccharides also happened to be the largest molecular size fractions.

Analytical methods. Phosphate was determined by the method of Chen *et al.* (16), and protein by the method of Lowry *et al.* (17). Free sialic acid was estimated by the thiobarbiturate method of Aminoff (18), and the total sialic acid content was measured by the method of Svennerholm (19). Neuraminidase (*V. cholerae*) treatment of the group B conjugates was carried out as previously described for the group B polysaccharide (20). ^{13}C nmr (nuclear magnetic resonance) spectroscopy was carried out on a Varian CFT20 spectrometer (in 10-mm tubes) operating at 20 MHz in the pulsed Fourier transform mode with complete proton decoupling. Chemical shifts are reported in parts per million (ppm) downfield from external tetramethylsilane, and the ^2H resonance of deuterium oxide was used as a field frequency lock signal.

Depolymerization of the group C polysaccharide. The group C polysaccharide (100 mg) was depolymerized by dissolving it in water (10 ml), adjusting the pH of the solution to 7.2 with 0.1 M NaOH, and heating the resultant solution at 100°C for 4.5 hr. The solution was then cooled and lyophilized, and the mixture of oligosaccharides was applied to a Sephadex G-50 column equilibrated with pyridine-acetate buffer at pH 5.4. The broad highest m.w. peak was lyophilized to yield 70 mg of a mixture of oligosaccharides, which were further fractionated using a Sephadex G-25 column equilibrated with the same buffer. The 1st fraction was collected and lyophilized to yield 50 mg of oligosaccharides in the m.w. range of 2500 to 3000. The fact that some of the *O*-acetyl groups of the group C polysaccharide (20, 21) were still retained on the resultant oligosaccharides was ascertained from their ^{13}C nmr spectra in which the methyl signal of the *O*-acetyl groups at 21.7 ppm (20, 21) was still observable. Variation in the time of heating of the group C polysaccharide produced varying degrees of depolymerization, thus providing a method for the controlled acid hydrolysis of this extremely acid-sensitive polysaccharide.

Reduction of the group A polysaccharide. The group A polysaccharide (average m.w. 25,000) (200 mg) was dissolved in 20 ml of water to which 100 mg of $NaBH_4$ was added. The reduction was left overnight at room temperature and was then dialyzed against water, and dialysate was subsequently lyophilized to yield 180 mg of the reduced group A polysaccharide.

Selective periodate oxidation of the polysaccharides. Selected molecular size fractions of the native group B (average m.w. 10,000), group C (average m.w. 40,000), and the reduced group A (average m.w. 25,000) polysaccharides were oxidized with 100 mM $NaIO_4$ solution (10 mg polysaccharide/1 ml) at room temperature in the dark for 15 min. Following this period, 2 ml of ethylene glycol were added to expend the excess $NaIO_4$, and the solution was left at room temperature for an additional 60 min. The oxidized groups A and C polysaccharides were dialyzed, lyophilized, and purified by gel filtration (Sephadex G-100 column). The group B polysaccharide was purified by the direct application of the ethylene glycol-treated reaction mixture to a Sephadex G-25 column. All the oxidized polysaccharides were recovered in 85 to 90% yields, having undergone no significant diminution in molecular size from the original polysaccharide. Each oxidized polysaccharide had an identical elution volume to the latter on the same calibrated columns previously used for the m.w. determinations of the original native polysaccharides.

Direct conjugation of the polysaccharides with proteins. The oxidized polysaccharides (70 to 100 mg) were added to 5.0 to 7.5 mg of TT or BSA solutions in 1 to 2 ml of 0.25 M K_2HPO_4 (pH 9.0) for the group A polysaccharide, and 0.75 M K_2HPO_4 (pH 9.0) for the groups B and C polysaccharides. The variation in the ionic strength of the buffer was used to suppress the formation of insoluble precipitates formed by ionic associations between the polysaccharides and the proteins. Sodium cyanoborohydride (20 to 40 mg) (Aldrich, Milwaukee, WI) was added to the solutions, followed by 3 drops of toluene, and the reaction mixtures were magnetically stirred in sealed vials for 11 to 13 days at 40°C. The reaction mixtures were then applied directly to Sephadex G-100 columns, and the highest m.w. fraction of each was collected and concentrated by ultrafiltration. The concentrated conjugates were then analyzed for their polysaccharide and protein contents.

Using similar procedures to those described above, the oxidized group B polysaccharide was also conjugated to ϵ-lysine, and the native group B polysaccharide was conjugated directly to BSA via the hemiketal of its reducing end-group sialic acid residue. The above conjugates were purified on Sephadex G-25 and G-100 columns, respectively. Treatment of the group B conjugates, linked through their terminal nonreducing sialic acid residues, with neuraminidase failed to liberate any free sialic acid from them, in contrast to the native group B polysaccharide when similarly treated (21).

Indirect conjugation of the group C oligosaccharide with BSA. The arylamine coupling reaction was carried out using thiophosgene essentially as described by McBroom *et al.* (22). In preparation for this reaction, the 2-(4-aminophenyl)-ethylamine derivative of the oligosaccharide was prepared (23). The oligosaccharide (50 mg) was dissolved in 1 ml of water and added to a rapidly stirred solution, adjusted to pH 8.0, of 1 ml of 2-(4-aminophenyl)-ethylamine and sodium cyanoborohydride (25 mg) in 1 ml of water. The reaction was allowed to proceed at room temperature for 3 days, during which time the pH was kept constant (pH 8.0) by automatic titration with 0.1 M NaOH. The reaction mixture was then applied directly to a Sephadex G-25 column equilibrated with 0.02 M pyridine acetate buffer at pH 5.4,

[3] Abbreviations used in this paper: EDC, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride; TT, tetanus toxoid; PBS, 0.01 M phosphate buffered physiologic saline; HSA, human serum albumin; ELISA, enzyme-linked immunosorbent assay.

and the first-eluted fraction, which contained both sialic acid and arylamine (absorbance at 236 nm) substituents, was lyophilized.

The above derivative was dissolved in 70% aqueous ethanol (6 ml), and 160 μl of thiophosgene were added slowly to the solution, maintaining a pH of 7.0 by titration with 1 M NaOH in 75% aqueous ethanol. When the consumption of base had ceased, the excess thiophosgene was expelled by repeated evaporation of methanol from the product. A solution of the above oligosaccharide isothiocyanatophenyl derivative in PBS (2 ml) at pH 9.0 was added to a stirred solution of BSA (10 mg) in 6 ml of the same buffer. Coupling was then allowed to proceed for 72 hr at room temperature, maintaining a constant pH of 9.0 by the automatic titration of the reaction mixture with 0.01 M NaOH solution. The reaction mixture was then applied directly to a Sephadex G-100 column (1.6 x 30 cm), and the 1st-eluted fraction, which contained both sialic acid and protein, was concentrated by ultrafiltration.

Immunization procedures. New Zealand White rabbits (approximate weight 2 to 3 kg) were immunized in the footpads with 30 to 50 μg of the selected fractions of the groups A, B, and C native polysaccharides, and their respective TT conjugates suspended in Freunds' complete adjuvant (Difco, Detroit, MI). Two immunizations were given 21 days apart, and the rabbits were bled 7 to 11 days after the 2nd injection. Groups of 50 Swiss white mice were given 5 subcutaneous injections at weekly intervals with the same polysaccharides and their respective conjugates (5 μg in 0.2 ml PBS), and 7 days after each injection 5 mice from each group were exsanguinated.

Immunodiffusion. Double radial immunodiffusion was performed in 0.8% agarose gels in PBS containing 2% polyethylene glycol (m.w. 4000) (24).

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed by a modification of the method of Voller *et al.* (25) in polystyrene microtiter plates (Linbro Chemical Co.), and the reagents were prepared using the following methods. Mouse IgG antibody was purified from normal mouse serum by precipitation with ammonium sulfate, followed by chromatography on a DEAE cellulose column (26). Specific rabbit anti-mouse IgG antibody was obtained by affinity chromatography. The IgG fraction of rabbit anti-mouse IgG (Miles-Yeda, Ltd., Rehovot, Israel) was passed through a Sepharose 4B column to which pure mouse IgG was covalently bound by activation with cyanogen bromide (27). Specific antibody was eluted from this column with a 0.2 M glycine-HCl buffer (pH 2.8). Pure antibody was conjugated to alkaline phosphatase (Miles Laboratories (PTY) Goodwood, South Africa; 1061 U/mg protein) by the addition of glutaraldehyde (28) according to Engvall *et al.* (29). The enzyme-IgG complex was separated by gel chromatography on a Sepharose 6B column (1.6 x 30 cm) equilibrated with PBS. To the highest molecular size fraction eluted from the void volume of the column was added 5% human serum albumin (HSA) for stabilization of the reagent. The reagent was then stored at 4°C with 0.02% sodium azide as a preservative. Each reagent was added to the wells in a volume of 0.2 ml. The wells were coated using a 5 μg/ml solution of each antigen in 0.05 M sodium carbonate-bicarbonate buffer at pH 9.6 at 37°C for 3 hr and a further 16 hr at 4°C. The plates were washed 5 times with PBS containing 0.05% Tween 20 at this time and then subsequently between the addition of each additional reagent. After the 1st wash, the wells were filled with serial 10-fold dilutions of mouse antisera (immune and preimmune), and the plates were left for 24 hr at 4°C. The initial dilution of the antisera was 1:100. After a further wash, alkaline phosphatase conjugated with rabbit-anti-mouse IgG (4 μg/ml PBS-0.05% Tween), previously purified by affinity chromatography, was added. The above solution was left in the microtiter plates overnight at room temperature, and after washing, *p*-nitrophenylphosphate (1.5 mg/ml in 0.05 M sodium carbonate-bicarbonate buffer containing 1 mM $MgCl_2$) was added to each well. Color development was stopped at 100 min, the solutions were transferred to tubes containing 0.4 ml of 0.2 M NaOH solution, and the enzyme activity was measured as an increase in optical density at 400 nm. The values for serum, antigen, and buffer blanks were subtracted from the above measurements. The antiserum to the group C-TT conjugate used in the ELISA experiment contained 250 μg antibody protein/ml specific for the group C polysaccharide as measured by microprecipitin techniques described below.

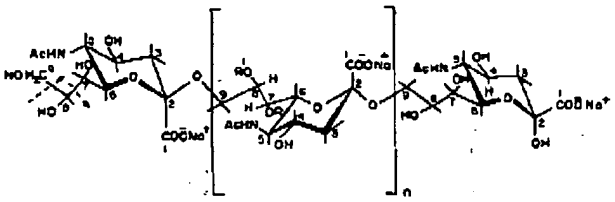
Quantitative microprecipitin experiments. These experiments were carried out according to the method of Kabat and Mayer (30) using 0.1 ml of antiserum (5 times diluted) and 0.1 ml antigen solution containing 1 to 50 μg of antigen. Precipitates were dissolved in 0.22 ml of 0.1 M NaOH, and the protein was determined by the method of Lowry *et al.* (17). For inhibition experiments, the antisera were first incubated for 1 hr at 37°C with increasing amounts of inhibitor before the addition of the antigen solution. The amount of antigen used was determined from its respective precipitin curve at equivalence point.

Bactericidal assays. These were carried out by the microbactericidal procedure described by Frasch and Chapman (31).

RESULTS

The introduction of a reactive aldehyde group into the polysaccharide. Because of the unique structures of the meningococcal groups B and C polysaccharides (20, 21), it was possible by means of controlled periodate oxidation to introduce into their structures a terminally located aldehyde group. The structure of the group C polysaccharide is depicted in Figure 1, and although one might expect some of its internal C7–C8 bonds to be readily cleaved by periodate oxidation, in actual fact the oxidation of the vicinal 7- and 8-hydroxyl groups of the 9-linked sialic acid residues is extremely slow (20). In addition, the polysaccharide is further stabilized by O-acetyl substituents strategically located on many of these same vicinal hydroxyls. However, the oxidation of the same 7- and 8-hydroxyl groups of the unlinked terminal nonreducing sialic acid residue is extremely fast (32) and probably generates an aldehyde group at C7 of this residue. It is unlikely that the reducing terminal sialic acid residue would oxidize to any great extent under these conditions because it exists in solution mainly in its pyranose ring form (20) and as such should behave similarly to an interchain residue.

The structure of the group B polysaccharide (20, 21) is shown in Figure 2 and has properties in relation to its periodate oxidation similar to those of the group C polysaccharide except that the interchain 2 → 8-linkages make the interchain sialic acid residues even more stable. Thus the aldehyde group is also generated at C-7 of the nonreducing end-group sialic acid residue of the group B polysaccharide. The structure of the group A polysaccharide (21, 33) presents a different situation to both the group B and group C polysaccharides, in that its nonreducing *N*-acetylmannosamine end-group is no more susceptible to periodate oxidation than any of the non-O-acetylated internal residues. However, the reducing end-group *N*-acetylmannosamine residue can be made into the most susceptible residue by simply reducing it to its open chain *N*-



1. R = R' = H
2. R = R' = $COCH_3$
3. R = H, R' = $COCH_3$
4. R = $COCH_3$, R' = H

Figure 1. Structure of the meningococcal group C capsular polysaccharide depicting the terminal sialic acid residues.

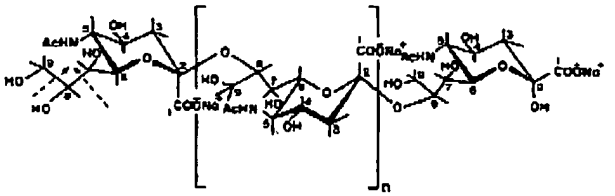
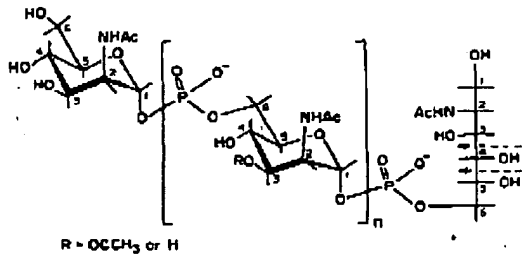


Figure 2. Structure of the meningococcal group B capsular polysaccharide depicting the terminal sialic acid residues.



R = $OCCH_3$ or H

Figure 3. Structure of the reduced meningococcal group A capsular polysaccharide depicting the terminal *N*-acetylmannosamine and *N*-acetylmannosaminitol residues.

acetylmannosaminitol derivative, as shown in Figure 3. In this form the modified group A polysaccharide can be selectively oxidized at this residue to generate a terminally located aldehyde group. Gel filtration of all the above polysaccharides before and after oxidation indicated that oxidation had caused no significant diminution in the molecular sizes of these polysaccharides, and therefore that only minimal interchain breakage could have occurred (Table I).

Depolymerization of the group C polysaccharide. Heating a neutral (pH 7.2) solution of the group C polysaccharide at 100°C resulted in a controlled depolymerization of this extremely acid-sensitive polysaccharide. Even after 4.5 hr of this treatment, the average molecular size of the major fraction of oligosaccharides was still in the range of 2500 to 3000. Therefore, increasing or decreasing the time of this treatment resulted in a predominance of smaller oligosaccharides or larger m.w. depolymerized polysaccharides, respectively. It is interesting to note that the *O*-acetyl groups of the original group C polysaccharide (20, 21) were retained on the depolymerized products, although the amount retained was found to be dependant on the time of heating. Similar depolymerizations have also been accomplished using the phosphorylated group A polysaccharide with this technique. A similar process was first used to depolymerize the more acid-stable pneumococcal type III polysaccharide (34), but the above experiments indicate the even greater versatility of this technique in its ability to perform controlled depolymerizations of very highly acid-sensitive polysaccharides.

Conjugation of polysaccharides and oligosaccharides to proteins. The polysaccharides, modified polysaccharides, and oligosaccharides were conjugated to BSA and/or TT by means of different methods. The group C oligosaccharide was coupled to BSA by an indirect process involving the preliminary addition of 2-(4-aminophenyl)-ethylene to the hemiketal of its reducing terminal sialic acid residue. This was accomplished by reductive amination, thus converting the terminal sialic acid residue into an open chain spacer (23). The above compound was treated with thiophosgene in 70% ethanol to yield its isothiocyanatophenyl derivative, which could then be coupled directly to BSA by the method of McBroom *et al.* (22). The purified conjugate contained 20% sialic acid, thus indicating that it was composed of a molar ratio of oligosaccharide to BSA of approximately 5.5:1.0. The yield of conjugate, as in the case of all the conjugates described in this paper, was in excess of 90% in terms of the total protein content.

TABLE I

Conditions of Formation and Analysis of the Group A, B, C Polysaccharide-Protein Conjugates

Polysaccharide	M.W. of Polysaccharide Chains for Conjugation	Molar Ratio of Polysaccharide to Protein	Time of Reaction	Molarity of Phosphate Buffer at pH 9.0	Molar Ratio of Polysaccharide to TT in Conjugate	Molar Ratio of Polysaccharide to BSA in Conjugate
			days			
Group A Reduced and Oxidized	2.5×10^4	48:1	12	0.25 M	0.4:1.0[a]	
Group B Native	1.0×10^4	68:1	11	0.75 M		0.7:1.0[c]
Group B Oxidized	1.0×10^4	68:1	11	0.75 M	2.0:1.0[b]	2.5:1.0[c]
Group C Oxidized	4.0×10^4	35:1	13	0.75 M	1.1:1.0[b]	

[a] Determined by gel-filtration on Sephadex G-100 or G-50 before and after oxidation.
[b] Determined from its phosphate/protein ratio.
[c] Determined from its sialic acid/protein ratio.

The above method could not be used with the higher m.w. polysaccharides (Table I) because of their tendency to precipitate in 70% ethanol solution during the formation of the isothiocyanatophenyl derivative. Therefore, a direct coupling to protein of these polysaccharides by reductive amination, in aqueous solution, through their terminal hemiketal groups, was attempted. The group B polysaccharide was coupled directly to BSA by this method and yielded a conjugate containing only 10% sialic acid, which indicated a molar ratio of group B polysaccharide to BSA of approximately 0.7:1.0. Modification of the above procedure by first incorporating a terminally located aldehyde group in the polysaccharide molecules and using this more reactive group in the reductive amination procedure proved to be more successful. Using identical conditions to those employed above the modified group B polysaccharide, containing a terminally located aldehyde group, yielded a 4-fold increase in the amount of coupled polysaccharide (Table I). Thus, the molar ratio of group B polysaccharide to BSA in this case was 2.5:1.0. This latter method was finally employed to couple the groups A, B, and C, polysaccharides to TT, and the final composition of the purified conjugates is shown in Table I. Probably because of its higher m.w., the group C polysaccharide was coupled less efficiently than that of group B polysaccharide. However, other factors in addition to molecular size are also involved in the efficiency of this coupling, because the group A polysaccharide, intermediate in molecular size to the groups B and C polysaccharides, was coupled the least efficiently on a molar basis.

Immunologic properties of the meningococcal conjugates: Immunization of rabbits. The groups A, B, and C polysaccharide-TT conjugates were used as immunogens in rabbits, and the antisera were evaluated by quantitative precipitin and immunodiffusion analysis. The precipitin curves obtained from each of the above antisera, when treated with its homologous TT conjugates, indicated that a good antibody response was obtained using all 3 TT conjugates. This was also confirmed by immunodiffusion experiments shown in Figure 4, where each of the TT conjugates also gave a strong precipitate with its homologous antiserum. In addition, this latter analysis was also able to further differentiate the antibody response and to identify antibodies of differing specificities in each conjugate. The groups A-TT and C-TT conjugates gave similar results, in that both yielded antisera that gave a precipitin line with both their homologous conjugates and their respective homologous polysaccharides. Spurring between these lines in the case of A also indicated that antibodies specific for TT were also present.

The group B polysaccharide-TT conjugate antiserum proved to

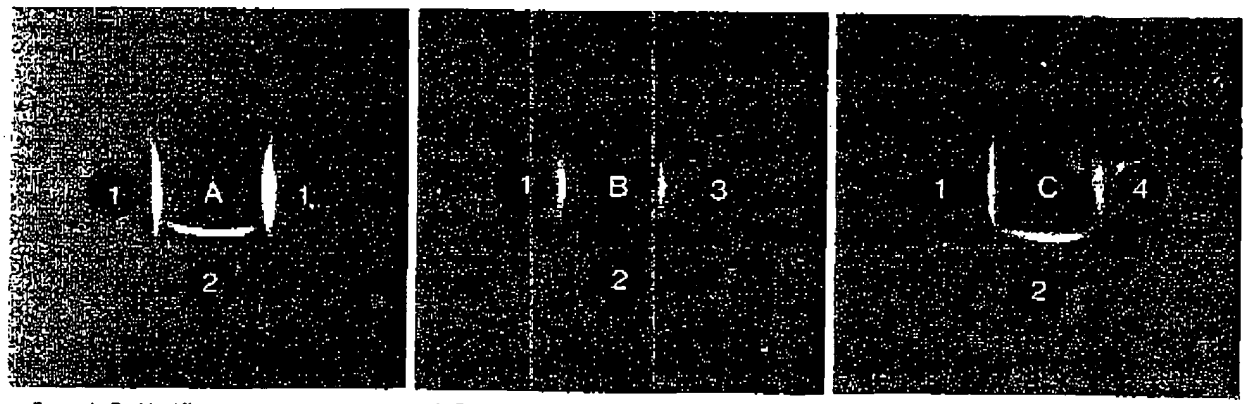


Figure 4. Double diffusion in agar of the anti-group A, B, and C polysaccharide-TT conjugate rabbit sera (wells A, B, and C) with their respective homologous conjugates (well 1), their respective native capsular polysaccharides (well 2), a group B polysaccharide-BSA conjugate (well 3), and a 2 → 9 *o*-linked sialic acid oligosaccharide-BSA conjugate (well 4).

be an exception to the above examples, because although it precipitated with the homologous conjugate, it gave no precipitin line with the homologous polysaccharide. However, the fact that antibodies with specificities for other than TT were present was indicated by the fact that a similarly linked group B polysaccharide-BSA conjugate also gave a strong precipitin line with the use of the same antiserum. This result suggested that the determinant responsible for the production of these latter antibodies was situated at the common linkage site (lysine to C7 of the terminal nonreducing heptulosonic acid residue) of both conjugates. This was confirmed by inhibition experiments shown in Figure 5, when a similarly linked group B polysaccharide-lysine conjugate proved to be the most powerful inhibitor of the anti-group B polysaccharide-TT conjugate serum-group B polysaccharide-BSA conjugate system. The ability of the oxidized polysaccharide to inhibit the above precipitation more efficiently than the native group B polysaccharide is also consistent with the above evidence. The fact that the native polysaccharide does inhibit the above precipitation, albeit weakly, also indicates the presence of antibodies that retain some group B polysaccharide specificity. In all cases (groups A, B, and C), the control anti-polysaccharide rabbit serum failed to precipitate with its homologous polysaccharide.

Both the native group C (20, 21) and group A (21, 33) polysaccharides contain O-acetyl substituents, the locations of which have been previously established and are shown in Figures 1 and 3, respectively. Comparative precipitin tests using both the native and de-O-acetylated groups A and C polysaccharides with their homologous TT-conjugate sera were performed to determine the role played by these substituents in the serology of the conjugates. These results are shown in Figure 6 and show that the de-O-acetylated C polysaccharide precipitated much less of the total antibody (0.5 mg/ml) than the native group C polysaccharide (2.0 mg/ml). These results are consistent with both the retention of O-acetyl substituents on the native group C polysaccharide moiety of the group C-TT conjugate and the relative importance of these substituents in the serology of the native group C polysaccharide. In contrast, the de-O-acetylated group A polysaccharide precipitated more of the total antibody (2.1 mg/ml) to the group A-TT conjugate than the corresponding native group A polysaccharide (1.7 mg/ml). This reversal, when compared with the results obtained with the group C polysaccharide, can be best explained by the fact that the original group A-TT conjugate contained no O-acetyl substituents. These groups, being extremely base labile, were probably lost during the reduction of the reducing end-group *N*-acetylmannosamine residue of the native group A polysaccharide. This reduction normally generates a fairly basic solution (>pH 10). Also in contrast to the group C-TT conjugate, both the native and de-O-acetylated group A polysaccharides precipitated fairly comparable amounts of the total antibody, thus indicating a more minor role played by these substituents in the serology of the group

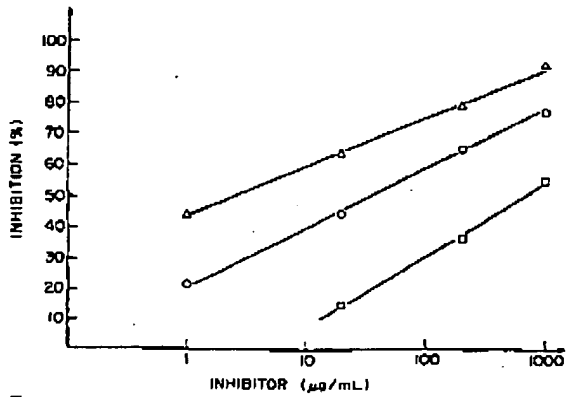


Figure 5. Inhibition of the precipitation of the anti-group B polysaccharide-TT conjugate rabbit serum by a group B polysaccharide-BSA conjugate (equivalence point 0.95 mg protein/ml antiserum) using a group B polysaccharide-lysine conjugate (Δ), the periodate oxidized group B polysaccharide (○), and the native group B polysaccharide (□).

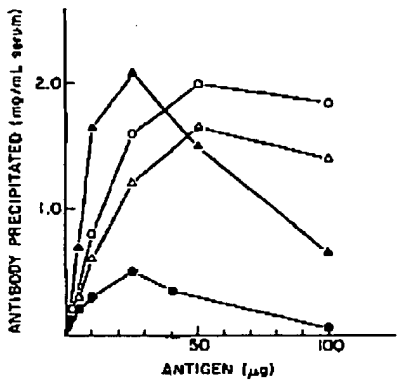


Figure 6. Quantitative precipitin analysis of the native (○——○), and de-O-acetylated (●——●) group C polysaccharide and the native (Δ——Δ), and de-O-acetylated (▲——▲), group A polysaccharide with the rabbit antisera to their respective TT-conjugates.

A polysaccharide. Although the group B-TT conjugate antiserum reacted with its homologous conjugate, no precipitate was detectable with the native group B polysaccharide used in precipitin experiments similar to those described above.

Immunization of mice. The groups A, B, and C polysaccharides and their TT-conjugates were subcutaneously injected in mice at weekly intervals, and 7 days after each injection the sera were evaluated for antibody (IgG) levels to the conjugates, using the homologous polysaccharide-TT conjugates as coating antigens in the ELISA technique (25). Although the sera of the mice repeatedly immunized with the polysaccharides showed no significant increase in antibody level over that of the preimmune sera, the sera of the mice immunized with each of the conjugates had attained substantially increased antibody levels after the 3rd immunization. The evaluation of 1 typical serum from a mouse injected with the group C-TT conjugate is shown in Figure 7. This particular serum was evaluated by coating the wells with the homologous conjugate, the homologous native polysaccharide, and a 2 → 9 α-D-linked sialic acid oligosaccharide-BSA conjugate. The reciprocal end-point titers indicate that there is a marked increase in the titer of the post-immune serum over that of the preimmune serum. The relatively higher titer associated with the group C-TT conjugate (Fig. 7) over that of its native polysaccharide is consistent with the formation of antibodies associated with the TT moiety. It is interesting to note that although the group C native polysaccharide was as efficient as a coating antigen in the wells of the polyvinyl plates as its homologous oligosaccharide-BSA conjugate, the group A polysaccharide could not be used as a direct coating antigen.

After the immunization of mice with the group B-TT conjugate, ELISA assays were done on the pooled sera of the groups of 5 mice bled after the 2nd, 3rd, and 4th injections. These results are shown in Figure 8 and largely corroborate results obtained in previous experiments using the rabbit antiserum to the same conjugates in which no antibody to the native group B polysaccharide could be detected. This is exemplified by the failure of the group B-BSA conjugate, linked through its terminal reducing sialic acid residue, to give any significant titer to this antiserum, whereas a similar group B-BSA conjugate, linked through its terminal nonreducing heptulosonic acid residue, presumably via a lysine residue of BSA, gave a relatively high titer. This result indicated that as in the case of the rabbit antibodies to the group B-TT conjugate, the linkage point between the terminal heptulosonic acid residue of the group B polysaccharide and a lysine residue of TT is a strong determinant in the production of mouse antibodies. The homologous group B-TT conjugate gave increasing titers with repeated injections of the mice, each of these titers being relatively higher than those obtained on the antisera with the similarly linked group B-BSA conjugate. This evidence indicates the presence of antibodies with a specificity for TT in the mouse antiserum. It is of interest to note that after the 2nd injection the difference between these 2 titers was at a maximum, the group B-BSA conjugate giving only a minimal titer. This implied that the antibodies with a speci-

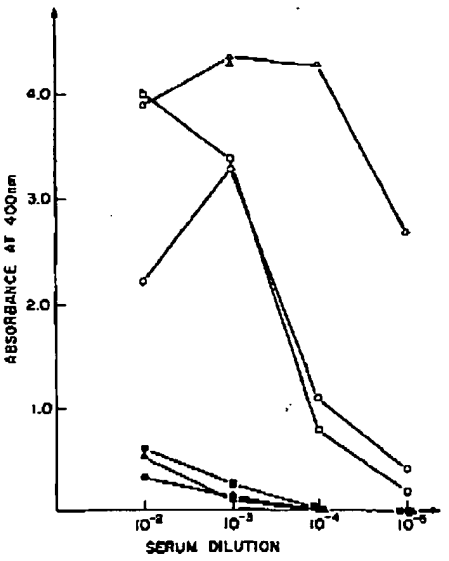


Figure 7. Titration of anti-group C polysaccharide-TT conjugate mouse serum in ELISA against different antigens. The wells were coated with the homologous conjugate (△), the homologous group C polysaccharide (□), and a 2 → 9 α-D-linked sialic acid oligosaccharide-BSA conjugate (○). Titration curves of the preimmune serum with the same antigens are marked with identical but solid symbols (▲, ■, ●).

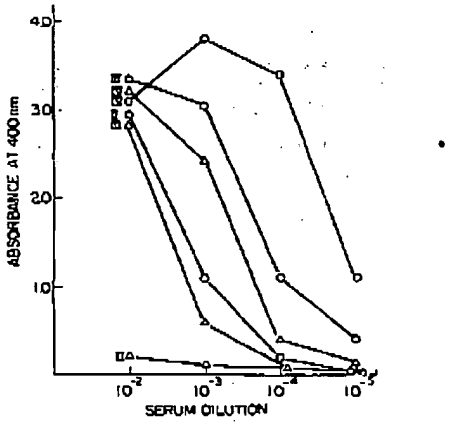


Figure 8. Titration of the anti-group B polysaccharide-TT conjugate mouse serum in ELISA using the homologous group B-TT conjugate (○——○) and the similarly linked group B-BSA conjugate (△——△) as coating antigens no measurable titer could be obtained using the group B-BSA conjugate linked through its reducing end-group as a coating antigen. The *roman numerals* depict the number of weekly injections required to give the corresponding titration curves.

ficity for TT are developed at a faster rate than those having the linkage specificity.

Bactericidal assays of the mouse antisera produced by the subcutaneous injection of mice with the groups A and C polysaccharide-TT conjugates demonstrated the development of significant bactericidal activity after the 3rd injection. Although the preimmune sera gave no significant bactericidal titer, the group A-TT conjugate induced in the mice a titer of 1/64, whereas the group C-TT conjugate induced in the mice a titer of 1/4096. No bactericidal activity was detected in the mouse antisera produced to the group B-TT conjugate.

DISCUSSION

The use of a simple and direct coupling procedure would be a decided advantage in the preparation of polysaccharide-protein conjugates for potential use as human vaccines. However, in our earlier unpublished experiments this approach with both cyanogen bromide (35) and EDC (12) used to couple the group C polysaccharide directly to BSA had proven to be unsatisfactory, due either to the poor incorporation of the polysaccharide or to the formation of water-insoluble complexes. This latter phenomenon could be attributed to cross-linking, facilitated by the activation of too many functional groups per polysaccharide molecule, although Beuvery *et al.* (6) have recently reported some success in coupling the group C polysaccharide to TT by using EDC despite the random native of this approach to the activation of the carboxylate groups. One method of avoiding this situation would be to insert a functional group into the polysaccharide molecules that had different chemical properties to those groups already residing on the polysaccharide. The ideal situation would be to specifically introduce 1 of these functional groups per polysaccharide molecule, preferably located in a terminal position. An even simpler application of this approach, which would require no modification of the polysaccharide, would be to utilize the potential keto-group of the terminal sialic acid residue of the groups B and C polysaccharides. This functional group could then conceivably be conjugated directly to proteins by reductive amination by using sodium cyanoborohydride (36). Certainly this reagent is acceptable for accomplishing these particular conjugations because it has been successfully used to conjugate oligosaccharides to protein via the hemiacetal groups of their terminal reducing sugar residues (13) and has the advantage of functioning at pH conditions mild enough to permit the retention of labile substituents (e.g., *O*-acetyl). It also has the added advantage of forming a tolerable linkage in terms of its possible use in the synthesis of human vaccines.

Certainly similar reduced Schiff base structures have already been identified in animal tissue (37). However, although reductive amination efficiently coupled fairly large R-type meningococcal oligosaccharides (m.w. 1500) (38) through their reducing terminal KDO residues (H. J. Jennings and C. Lugowski, manuscript in preparation) directly to protein, the use of the same procedure on the larger molecular size group B polysaccharide (m.w. 10,000) proved to be much less efficient. Therefore, in order to activate the higher molecular size polysaccharide to this reagent, a more reactive terminal free aldehyde group was introduced into their structures, thus forming potentially monovalent polysaccharide molecules. This was achieved by the time-controlled periodate oxidation of the native groups B and C polysaccharides and of the reduced group A polysaccharide. The unique structures of the above polysaccharides were conducive to the preferential cleavage of only terminally located vicinal hydroxyl groups. In the case of the groups B and C polysaccharides, the aldehyde was introduced at C-7 of their nonreducing terminal sialic acid residues, and in the group A polysaccharide probably at C-5 of its reduced terminal *N*-acetylmannosaminitol residue.

The protein chosen as the standard carrier in the preparation of conjugates from the above polysaccharides was TT because it is already approved as a human vaccine, as are both the groups A and C polysaccharides (1). Beuvery *et al.* (6) and Schneerson *et al.* (7) also utilized TT in the formation of their respective conjugates of the meningococcal group C polysaccharide and *H. influenzae* type b polysaccharide. The activated groups A, B, and C polysaccharides were conjugated to TT by reductive amination, and although the coupling proceeded fairly slowly, most of the criteria associated with an effective coupling were met. Serologic studies using these conjugates demonstrated that the *O*-acetyl substituents of the group C polysaccharide were retained by means of this coupling procedure, and also emphasized the importance of these substituents to the serologic properties of the native group C polysaccharide. In contrast to the group C polysaccharide, these serologic studies also provided evidence that the *O*-acetyl substituents normally associated with the native group A polysaccharide (21, 33) were not present on the group A polysaccharide-TT conjugate, and that these substituents also play a much smaller role in the serologic properties of the native group A polysaccharide. However, the loss of these substituents may not be entirely attributable to the coupling procedure, and it is more likely that the major loss occurred under the more alkaline conditions generated in the reduction of the native group A polysaccharide before its periodate oxidation. Obviously, in order to sensitize reducing end-

group sugar residues to periodate oxidation by means of this reducing technique, some measure of pH control over the reduction procedure will be required if one wishes to retain the alkali labile O-acetyl substituents. Except for the group A polysaccharide, those of groups B and C yielded conjugates with more than a 1:1 molar ratio of polysaccharide to TT, and the fact that this ratio was higher (2:1) in the case of the group B conjugate provides some evidence that the molecular size of the polysaccharide could be a factor in determining the final ratio of polysaccharide to TT in the resultant conjugate.

The serologic experiments using the above conjugates indicate that, with the exception of the group B polysaccharide, those of groups A and C had been successfully converted to thymic-dependent immunogens; in their conjugated forms the immunogenicities of both these polysaccharides were substantially increased in rabbits and mice over that of their native polysaccharides. In addition, the presence of antibodies with a specificity for TT were also detected in the antisera from the above experiments. The potential of the groups A and C polysaccharide-TT conjugates for use as human vaccines is best demonstrated in the serologic results obtained by their weekly subcutaneous injection in mice. After the 3rd injection, both the group A-TT and group C-TT conjugates had elicited the development of high-titer polysaccharide-specific antisera that also proved to be bactericidal for their respective homologous groups A and C organisms. It is interesting to note that the above favorable serologic properties were obtained using rather small molecular size polysaccharides to form the conjugates, indicating that conjugation of these polysaccharides may change the large molecular size dependency normally associated with the immunogenicity of the native groups A and C polysaccharides in humans (1). This observation has also been reported by Schneerson *et al.* (7).

The group B polysaccharide-TT conjugate proved to have unique serologic properties in comparison with the groups A and C polysaccharide conjugates. The immunogenicity of the group B polysaccharide was not enhanced by conjugation to TT, and no bactericidal activity to the homologous group B organisms could be detected. However, a fairly substantial antibody population with a specificity for other than TT was detected in the antiserum. This population of antibodies was shown by inhibition experiments to be highly specific for the linkage between the lysine residues of TT and the terminal heptulosonic acid residue of the oxidized group B polysaccharide, thus providing serologic evidence for the exact point of linkage between these 2 molecules. The failure of the group B polysaccharide to elicit an antibody response even in its conjugated form provides strong evidence for the structural identity-basis of its poor immunogenicity. The alternative hypothesis of the enzymatic (neuraminidase) degradation of the group B polysaccharide in animal tissue is not valid for this particular conjugate because in the conjugation process the normally neuraminidase-sensitive terminal nonreducing sialic acid residue of the group B polysaccharide (20) is modified and becomes stable to this enzyme. The recent identification of 2 → 8 α-O-linked sialic acid residues in human gangliosides (39) is also consistent with the former hypothesis and casts some doubt on the possible efficacy of a group B polysaccharide-TT conjugate for use as a human vaccine.

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INDUCTION OF AZOBENZENEARSONATE- (ABA) SPECIFIC HELPER AND SUPPRESSOR T CELLS AND *IN VITRO* EVALUATION OF THEIR ACTIVITIES IN THE ANTIBODY RESPONSE TO T-DEPENDENT ABA-PROTEIN CONJUGATES[1]

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The present study demonstrates the regulatory role of ABA-specific helper and suppressor T cells in the antibody response to T-dependent ABA-protein immunogens. *In vivo* experiments have demonstrated that immunization of BDF_1 mice with ABA coupled to mouse immunoglobulin G (ABA-MIgG) induce suppression of the anti-TNP response to a subsequent challenge with trinitrophenyl-ABA-keyhole limpet hemocyanin (TNP-ABA-KLH). Anti-TNP antibody affinity was 5-fold reduced in ABA-MIgG-primed mice, as compared with controls, owing to preferential suppression of high affinity PFC. Popliteal lymph node cells from ABA-KLH footpad-primed mice when cultivated *in vitro* with TNP-ABA-KLH give a sizeable anti-TNP PFC response. Spleen cells from ABA-MIgG-primed mice not only fail to give anti-TNP PFC response when stimulated *in vitro* with TNP-ABA-KLH, but also suppress the anti-TNP response of lymph node cells from ABA-KLH primed mice. The induction of suppressor cells is dependent on the dose of ABA-MIgG injected and is ABA specific. The T cell nature of suppressor cells was demonstrated by their enrichment after passage over anti-immunoglobulin-coated plates. Spleen cells from ABA-MIgG-primed mice also suppress the anti-ABA PFC response of lymph node cells from ABA-KLH-primed mice stimulated *in vitro* with TNP-ABA-KLH or ABA-KLH. Experiments with separated and recombined helper, suppressor, and B cells induced by ABA-protein conjugates indicate that ABA-primed B cells need to be present in culture to obtain suppression of the anti-TNP response. Therefore, the regulatory role of ABA-specific cell populations on the anti-ABA antibody response can be analyzed *in vitro*, and this experimental system should facilitate the dissection of the network components involved in the anti-ABA immune response.

The immune system can be viewed as a functional idiotypic network in which regulatory interactions are based on idiotype-anti-idiotype recognition (1). Several systems have been used to test the validity of the network hypothesis and to analyze the cellular elements and the soluble factors operating in network regulation (reviewed in 2).

The idiotypic regulation of the immune response to the hapten azobenzenearsonate (ABA)[3] has been extensively studied in recent years, and a vast body of information has been accumulated. Hopper and Nisonoff (3) have demonstrated that A/J mice immunized with ABA conjugated to keyhole limpet hemocyanin (ABA-KLH) produce anti-ABA antibodies, some of which (20 to 70%) bear a cross-reactive idiotype (CRI). This group has also shown that injection of anti-CRI in A/J mice, followed by hyperimmunization with ABA-KLH, selectively suppresses the expression of CRI^+ antibodies with only negligible impairment of the total anti-ABA response. Suppression of CRI^- antibody response is mediated by suppressor T cells which have been shown to produce suppressive factors with idiotypic or anti-idiotypic receptors (4). In a series of very elegant experiments Greene, Benacerraf, and coworkers (5–7) have analyzed the induction and suppression of delayed-type hypersensitivity (DTH) to ABA. Induction of DTH to ABA has been obtained by subcutaneous injection of ABA coupled to syngeneic cells (5), i.v. injection of $F(ab')_2$ fragments of anti-CRI antibodies, subcutaneous injection of anti-CRI, and i.v. injection of anti-CRI in mice pretreated with cyclophosphamide (6). ABA-specific suppressor T cells, induced by i.v. injection of ABA-syngeneic cells, produce idiotype-bearing, $I\text{-}J^-$ suppressor T cell factor, which induces 2nd-order suppressor T cells expressing anti-idiotypic receptors (7).

In this report we establish the conditions to obtain *in vitro* antibody response to ABA-protein conjugates. ABA-specific helper and suppressor T cells can be generated in BDF_1 mice depending

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[3] Abbreviations used in this paper: ABA, azobenzenearsonate; CRI, cross-reactive idiotype; EACA, ε-amino caproic acid; KLH, keyhole limpet hemocyanin; MIgG, mouse immunoglobulin G; TNBS, 2,4,6-trinitrobenzene sulfonic acid; HB, methyl-*p*-hydroxybenzimidate; BGG, bovine γ-globulin; OVA, chicken ovalbumin; PAB, para-azobenzoate.